ORIGINAL ARTICLE

Tissue Specific Effects of Chronic Sustained Hypoxia on Oxidative Stress: Role of Cilnidipine, a Dual L/N Type Calcium Channel Blocker

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Abstract:

Background: Blood flow, metabolic rate and oxygen requirements of an organ guide the extent of oxidative stress experienced by any tissue in response to chronic hypoxia. Currently cilnidipine is used in the management of hypertension and its antioxidant actions are gaining wide interest. *Aim and Objectives:* To evaluate the tissue specific effects of chronic sustained hypoxia with regards to oxidative stress in the context of cilnidipine. *Material and Methods:* Twenty four adult male Wistar strain albino rats were randomly assigned into four groups: group 1, control, normoxia (21% O_2); group 2, chronic hypoxia (CH) (10% O_2) for 21 days; group 3, normoxia + cilnidipine (Cil) for 21 days; group 4, chronic hypoxia + cilnidipine (CH+Cil) for 21 days. Following 21 days of intervention blood was collected and animals were sacrificed and liver, lung and heart were collected. Serum MDA and MDA in tissue homogenate of liver, lung and heart were estimated. *Results:* Our results demonstrate the elevated serum MDA levels in chronic hypoxia exposed rats (group 2). We also observed increased MDA in liver followed by lung and least in the heart in chronic hypoxia exposed rats (group 2). Treatment with cilnidipine reduced serum MDA and heart MDA levels in cilnidipine treated chronic hypoxia exposed rats (group 4). However cilnidipine did not have any influence on MDA levels in the liver and lung in same group of rats. *Conclusion:* The results demonstrate tissue specific effects of chronic sustained hypoxia with the highest oxidative stress observed in the liver followed by the lung. Although oxidative stress is also observed in the heart it is the least in comparison to the liver and the lung. Cilnidipine, a dual L/N type calcium channel blocker demonstrated beneficial antioxidant actions only in the heart supporting the cardioprotective role of cilnidipine.

Keywords: Chronic Hypoxia, Oxidative stress, Cilnidipine

Introduction:

Oxygen is indispensable for life. Accordingly the cells have evolved mechanisms to detect and to react to alterations in the oxygen levels in the microenvironment. Both low and high oxygen levels disturb oxygen homeostasis and endanger cell survival [1, 2]. Hypoxia is lack of oxygen at the tissue level. Hypoxia may be acute or chronic and sustained or intermittent. Irrespective of its type, hypoxia is associated with increased generation of free radicals and consumption of the antioxidants disturbing the oxidant/antioxidant balance in favor of oxidants [3, 4]. The generated free radicals damage the constituents that make up the cell like nucleic acids, proteins, lipids and carbohydrates causing oxidative stress [5].

Blood flow and oxygen requirements of different organs are not uniform. Additionally the antioxidant reserves in different tissues also vary [6]. Consequently oxidative stress induced by hypoxia may be tissue and organ specific. This

understanding may be of significance in the management of various clinical conditions resulting from occurrence or exposure to hypoxia e.g. Chronic Obstructive Pulmonary Disease (COPD), congenital heart disease, high altitude etc. Hence the present study was undertaken to explore the organ specific effects of chronic sustained hypoxia particularly liver, heart and lung with regards to oxidative stress.

Cilnidipine is a Calcium Channel Blocker (CCB) belonging to the dihydropyridine class. Cilnidipine is unique owing to its dual L/N type calcium channel blocking property [7]. It has also been demonstrated to have some antioxidant properties owing to its strong lipophilic nature. Cilnidipine due to its pharmacological profile is being widely used in the treatment of hypertension and its antioxidant actions have currently gained wide attention [8]. The present study also intends to explore the antioxidant property of cilnidipine and its impact on the chronic hypoxia induced oxidative stress of various organs.

Material and Methods:

Experimental Animals:

Twenty four adult male Wistar strain albino rats (*Rattus norvegicus*), weighing 180 to 250 g were procured from the animal house of BLDE (Deemed to be University). All the animals were maintained at 22-24^{0}C and exposed to 12 hours light/dark cycle with food and water made available to them *ad libitum.* The animals were allowed to adapt to the laboratory conditions for a week before the onset of the experimental protocol.

Ethical considerations:

Institutional Animal Ethics Committee (IAEC) clearance was obtained for the study (Ref: BLDE/BPC/641/2016-2017 dated 21.10.2016) and all the experimental procedures were done in accordance with the guidelines of Committee for the Purpose and Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Experimental groups:

The experimental animals were randomly assigned to one of the four groups as depicted in Table 1. The body weight of all rats was recorded on day 1 and after 21 days of intervention using electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). The rats of all groups were matched for weight at the onset of the experimental protocol.

Table 1: Experimental Groups of Rats (n=6 in Each Group)

Groups	Intervention
Group 1 (Control)	Vehicle (0.5% Na CMC) by oral gavage for 21 days
Group 2 (CH)	Chronic hypoxia (CH) for 21 days + Vehicle (0.5% Na CMC) by oral gavage for 21 days
Group 3 (Cil)	Cilnidipine (2mg/Kg body wt.) in 0.5% Na CMC by oral gavage for 21 days
Group 4 (CH+Cil)	Chronic hypoxia (CH) for 21 days + cilnidipine (2 mg/Kg body wt.) in 0.5% Na CMC by oral gavage for 21 days

CH-Chronic Hypoxia, Cil-Cilnidipine, CH+Cil- Chronic Hypoxia+Cilnidipine, Na CMC- Sodium Carboxymethyl Cellulose

Experimental Protocol:

The experimental protocol followed has been summarized in Fig 1.

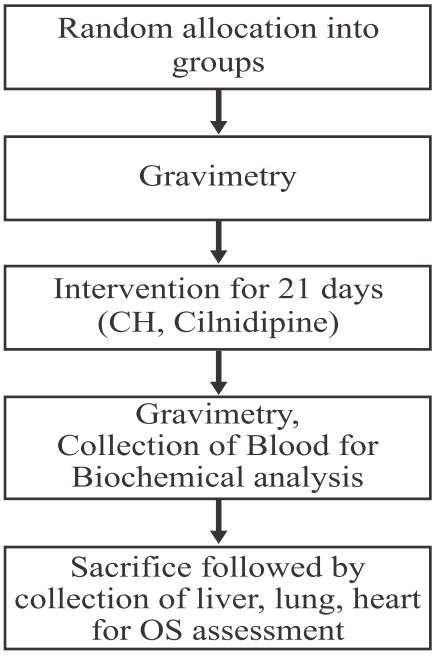

Fig 1: Experimental Protocol followed

CH=Chronic Hypoxia; OS=oxidative stress

Exposure of animals to Chronic (Normobaric) Hypoxia/CH:

Chronic sustained hypoxia was induced by placing caged rats (4 per cage) inside a acrylic chamber of 300-liter capacity, that can hold up to 4 cages (16 rats), and were exposed to inspired Oxygen (O_2) (10%) and Nitrogen (N_2) (90%) to induce normobaric hypoxia. The hypoxic environment was created with an inflow of mixture of room air and nitrogen. CO_2 was absorbed by soda lime (27 granules), and a desiccator removed excess humidity. The temperature was maintained at 22-26°C. The chamber was opened for 1 hour, two times a week to clean the cages and to replenish food and water [9]. In our study rats were exposed to chronic sustained hypoxia for a period of 21 days.

Administration of drug:

Cilnidipine is dual L/N type CCB. Cilnidipine was procured from Laksh Finechem Pvt. Limited, Gujarat, India. Cilnidipine was stored in the refrigerator until further use. The dose of cilnidipine for rats was calculated using the formula:

Rats (mg/kg) = Human dose×0.018×5 [10].

The dose was calculated to be 2 mg/kg body weight. A suspension of cilnidipine in 0.5% sodium Carboxymethyl Cellulose (0.5% Na CMC) was freshly prepared every day and was administered by oral gavage once daily in the morning to group 3 (Cil) and group 4 (CH+Cil) rats for 21 days.

Assessment of oxidative stress

Malondialdehyde is a product of lipid peroxidation and a frequently used marker of oxidative stress. MDA concentration (conc.) was estimated in the serum and tissue homogenate of liver, lung, and heart by the method of Buege and Aust (1978) [11]. 10% tissue homogenate was prepared in 0.1M phosphate buffer using tissue homogenizer (REMI MOTORS, Bombay, India) and supernatant was used for the assay. MDA reacts with Thiobarbituric Acid (TBA) to give a pink colour and the absorbance of was read at 535 nm using spectrophotometer (Schimadzu UV 800, Schimadzu corporation, Japan).

Statistical Analysis:

All the statistical analysis was done using SPSS 16.0 (SPSS Inc., Chicago, USA). The parameters are presented as Mean ± SD. Statistical significance of data across multiple groups was analyzed using One-way analysis of variance (ANOVA) followed by Post hoc Tukey's multiple comparison test to determine significant difference between groups. A p-value < 0.05 was considered as statistically significant.

Table 2: Comparison of Biomarkers of Oxidative Stress in Serum and Tissue Homogenate among Groups (n=6 in Each Group)

Parameter	Group 1 (Control)	Group 2 (CH)	Group 3 (Cil)	Group 4 (CH+Cil)	p-value
Serum MDA (µmol/L)	1.95±0.53	3.39±0.70[a]	1.48±0.15[b]	2.13±0.29[b]	0.000*
Liver MDA (µmol/gm of tissue)	27.27±3.68	43.68±3.50[a]	28.36±3.94[b]	41.31±1.81[ac]	0.000*
Heart MDA (µmol/gm of tissue)	10.79±0.51	13.89±0.59[a]	11.18±0.63[b]	11.66±0.83[b]	0.000*
Lung MDA (µmol/gm of tissue)	27.01±2.97	37.12±2.57[a]	28.88±3.33[b]	31.74±5.40	0.003*

*Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. Superscript a, b, c indicate significant difference between groups. 'a' denotes comparison with Group 1, 'b' denotes comparison with group 2, 'c' denotes comparison with group 3. *p<0.05. CH-Chronic Hypoxia; Cil-cilnidipine; CH+Cil- Chronic Hypoxia+Cilnidipine; MDA-Malondialdehyde*

Results:

Table 2 shows significant increase in serum MDA levels in chronic hypoxia exposed rats (group 2) compared to control rats (group 1). Also MDA levels in tissue homogenate of liver, heart and lung were significantly increased in chronic hypoxia exposed rats (group 2) compared to control rats (group 1). There was a 60% increase in liver MDA, 37% increase in lung MDA, 28% increase in heart MDA in chronic hypoxia exposed rats (group 2) compared to control rats (group 1). Chronic hypoxia exposed cilnidipine treated rats (group 4) did not demonstrate significant differences in liver MDA and lung MDA levels when compared to group 2 rats. However serum MDA, heart MDA were significantly reduced in group 4 (CH+Cil) compared to group 2 (CH).

Discussion:

The blood flow, oxygen requirements and antioxidant reserves in different tissues varies. Consequently there may be differences in the susceptibility of tissues to oxidative stress [6].

MDA is product of lipid peroxidation and has been used as an indirect biomarker of oxidative stress [11]. In the present study we assessed oxidative stress by estimating MDA levels both in the serum and tissue homogenate of liver, heart and lung to explore the organ specific effects of chronic hypoxia with regards to oxidative stress. We observed increased serum MDA levels in chronic hypoxia exposed rats compared to control rats indicating higher oxidative stress in the former. Among the tissues studied liver demonstrated the highest oxidative stress followed by the lung and least oxidative stress in the heart. This observation indicates that highest brunt of hypoxia is borne by the liver despite the fact that it has high antioxidant reserve. There are several factors contributing to this. Tissues like heart and brain are considered as

supply dependent and tissues like kidneys, skin, resting muscle and splanchnic area are considered as supply independent [12]. Whenever the body is challenged with any stressful condition like hypoxia, the blood flow is diverted to supply dependent vital organs like heart and brain at the expense of the visceral organs [13]. In addition, Reactive Oxygen Species (ROS) formation is decided by the levels of free iron, hemoproteins and Polyunsaturated fatty acids (PUFAs) all of them promoting its formation. Liver has more iron and free iron reacts with hydrogen peroxide via Fenton reaction producing hydroxyl radicals. Heme released from the hemoproteins also contributes to the formation of ROS via the same Fenton reaction. High levels of Cytochrome P450 in the liver have a role in the generation ROS [14]. Chronic hypoxia induced hepatic oxidative stress may play a vital role in the pathogenesis of liver diseases in patients experiencing hypoxia. Studies have reported higher prevalence of Nonalcoholic Fatty Liver Disease (NAFLD) in patients with Chronic Obstructive Pulmonary Disease (COPD) [15].

Lungs are the gate way for oxygen in the environment and the cells of the body. Accordingly lungs may be one of the early organs to experience adverse consequences of hypoxia. However, in the present study lung experienced lesser oxidative stress when compared to the liver. This could be due to tolerance of the alveolar epithelial cells to hypoxia due to which they can maintain adequate cellular ATP on exposure to prolonged hypoxia [16].

Cilnidipine is a dihydropyridine calcium channel blocker with dual L/N type calcium channel blocking property [7]. Cilnidipine is lipophilic and acts as a lipophilic chain breaking antioxidant. Among all the dihydropyridine derivatives cilnidipine demonstrates strongest lipophilicity and has highest antioxidant actions [8]. Although our study did not demonstrate any beneficial effects of cilnidipine on the oxidative stress levels in the liver and lung but was successful in reducing oxidative stress in the heart and serum MDA levels. This demonstrates the cardioprotective role of cilnidipine.

Implications of the study:

The results of our study demonstrate that liver is the one to suffer highest oxidative stress followed by the lung and the heart due to hypoxia. Hence the early markers of liver diseases should be monitored to identify their onset in patients experiencing hypoxia. Cilnidipine, a dual L/N type calcium channel blocker has been found to have a beneficial role to counteract hypoxia induced alteration of oxidant/antioxidant balance. Further study on the role of different antioxidant supplementation on disease progression can be undertaken.

Acknowledgements:

The corresponding author acknowledges BLDE (Deemed to be University) for providing a research grant for the study (BLDEU/REG/R&D /RGC/2017-2018/1593 Dated 23/12/2017).

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