Per cutaneous carbon dioxide mist treatment has protective effects in experimental myocardial infarction

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A R T I C L E   I N F O
Article history:
Received 8 January 2015
Received in revised form 9 March 2015
Accepted 25 March 2015
Available online 1 April 2015

Keywords:
Animal model
Carbon dioxide
Preconditioning
Mist
Myocardial infarction

A B S T R A C T

Per cutaneous treatment with carbon dioxide (CO2) mist, CO2 gas dissolved in water, contributes to improved cardiac function after myocardial infarction (MI). In this study, we investigated the effects of repeated pretreatment with CO2 mist on cardiac dysfunction after MI. The CO2 mist was generated by a dry mist production unit. The whole body of rats below the axilla was wrapped in a polyethylene bag, which was sealed and filled with the CO2 mist in the draft cabinet for 30 min daily for 7 days. MI was induced by ligation of the coronary artery in untreated (UT), CO2 gas-pretreated (CG), and CO2 mist-pretreated (CM) rats. The infarct size and the increase in oxidative stress due to MI were significantly smaller in the CM rats than in the UT rats. Furthermore, the expression of inflammation-related genes, such as monocyte chemoattractant protein-1, and fibrosis-related genes, such as transforming growth factor-β1, was significantly suppressed in the CM rats. The CM rats had a better left ventricular ejection fraction than the UT rats 7 days after MI. These parameters in the CG rats were the same as in the UT group. Thus, CO2 mist preparative treatment may be potentially useful for the reduction of MI.

1. Introduction

The cardioprotective effect of ischemic preconditioning was reported in 1986 (1). Ischemic preconditioning resulted in infarct sizes that were approximately 25% of those observed in untreated hearts. The original study not only demonstrated the cardioprotection from ischemia/reperfusion (I/R) injury, but also provided a model with which to study ways to protect the ischemic heart. There have been many subsequent reports of the cardioprotective effects of remote ischemic preconditioning (2–4), in which brief and repeated non-lethal ischemia and reperfusion of a remote organ or tissue can increase the heart’s tolerance of acute IR injury.

Balneotherapy in hot springs containing a high concentration of carbon dioxide (CO2) has long been applied clinically to treat a variety of diseases. The effect of CO2-enriched water on the cutaneous circulation depends primarily on the vasodilatation effects elicited by the CO2 that diffuses into the subcutaneous tissue through the skin layers (5,6). Previous studies have reported that CO2-enriched water induces peripheral vasodilatation, which increases cutaneous blood flow (6–8). Thus, bathing in CO2-enriched water may be a useful therapeutic method for patients with cardiovascular diseases. However, no artificial CO2-enriched water bathing system is widely available, probably because of the high setup cost and the difficulty of maintaining a constant artificial CO2 concentration.

Recently, instead of a CO2 bathing system, a new and simple device was developed to generate CO2 mist, i.e., water containing a
high concentration of dissolved CO2 molecules. We have previously reported that percutaneous treatment with CO2 mist produced by this device improved the development of cardiac remodeling after myocardial infarction (MI) (9). Treatment with CO2 mist also accelerated angiogenesis in a mouse model of peripheral arterial disease (10). However, it is still unclear whether pretreatment with CO2 mist has a cardioprotective effect against myocardial ischemic injury. In the present study, we investigated whether pretreatment with CO2 mist can reduce the infarct size in an experimental animal model of MI.

2. Materials and methods

2.1. Animals and experimental design

All procedures were performed in accordance with Osaka City University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The 8-week-old male Wistar rats weighing 260–290 g were purchased from CLEA Japan, Inc (Osaka).

The principal aim of the present study was to determine whether repeated pretreatment with CO2 mist can attenuate cardiac dysfunction after MI. After 24 h of the last treatment, MI was induced by permanent ligation of the left coronary artery (9,11,12) in rats that were either untreated (UT), or had undergone pretreatment with CO2 gas (CG) or CO2 mist (CM) daily for 7 days. Untreated and sham-operated rats were used as a control (CON) group. Forty-eight hours after MI induction, the infarct size was quantified by the Evans blue method (13). Three days after MI induction, the blood pressure and heart rate of the conscious rats were measured by the tail-cuff method (BP98A; Softron, Tokyo); a blood sample was then collected and the heart weight was measured. Seven days after MI induction, a transthoracic echocardiographic study was performed, and the rats were then sacrificed. The ventricle was separated into the upper and lower portions, and then the upper portion of the left ventricle was divided into the marginal and non-infarcted zones. The specimens obtained were immediately frozen in liquid nitrogen and stored at −80 °C until use. The lower portion of the left ventricle was fixed in 10% formaldehyde overnight and embedded in paraffin.

2.2. CO2 gas or CO2 mist treatment procedure

The CO2 mist was generated by a dry mist production unit (ACP JAPAN Co., Ltd., Tokyo) (9,10). In brief, 100% concentrated CO2 was compounded and compressed with water through dual fluid nozzles at 4 atm. The rat was placed on a heating plate at 37 °C, under anesthesia with sodium pentobarbital (40 mg kg−1, ip), and the whole body below the axilla was encased in a sealed polyethylene bag. The bag was then filled with CO2 mist or CO2 gas in the draft cabinet (9,10). The UT/MI and CON rats were kept on a heating plate (9,11,12). In brief, rats were anesthetized with pentobarbital. A twodimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles. The left ventricular (LV) ejection fraction (LVEF) was calculated by measuring the LV end-diastolic volume (LVEDV) and the LV end-systolic volume (LVESV), by using a modified Simpson’s method. Pulsed wave Doppler spectra (early rapid filling [E] wave and atrial contraction [A] wave) of mitral inflow velocities were recorded from the apical 4-chamber view, with the sample volume placed near the tips of the mitral leaflets and adjusted to the position at which velocity was maximum and the flow pattern was laminar, and the ratio of E wave velocity to A wave velocity (E/A) was calculated.

2.4. Histology and evaluation of oxidative stress

The area of interstitial fibrosis in the marginal area of the infarct was measured, as described previously (12). In brief, 4-μm-thick sections were cut and stained with hematoxylin-eosin stain.

At 3 days after MI induction, the serum levels of derivatives of reactive-oxygen metabolites (d-ROMs) were measured by the Free Radical Elective Evaluator (Diacon International, Grosseto, Italy) using commercial assay kits (Diacon International) (12). On the same day, cardiac oxidative stress was assessed by 8-hydroxydeoxyguanosine (8-OHdG) content, a marker of oxidative DNA damage, as described previously (15). Briefly, DNA from the LV tissues was extracted by Nal method using a DNA Extractor TIS Kit (Wako Pure Chemical Industries, Osaka, Japan). After 50 μg of DNA pellet was pretreated using 8-OHdG Assay Preparation Reagent Set (Wako Pure Chemical Industries), the 8-OHdG adducts were determined by use of an highly sensitive 8-OHdG enzyme-linked immunosorbent assay kit (Japan Institute for the Control of Aging, Shizuoka, Japan).

2.5. mRNA expression analysis

Total RNA in the marginal area of the infarct was extracted from tissues or exosomes with Isogen I (Nippon Gene, Toyama) (11,16). The concentration and quality of the RNA were assessed using a Nano Drop 2000 spectrophotometer (Thermo, Waltham, MA, USA). To quantify the gene expression levels, we used quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) with the TaqMan system (Applied Biosystems, CA, USA), as previously described. For normalization, the transcript levels were compared to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Western blot analysis

Our detailed method has been described previously (10,12). Protein extracts were obtained from homogenized left ventricles with no treatment or CO2 mist treatment for 7 days. After electrophoretic transfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA), the membranes were probed with each primary antibody. Antibodies were obtained from the following sources: anti-GAPDH and heat shock protein 72 (Hsp72) antibodies from Santa Cruz Biotechnology (Dallas, TX, USA); anti-phospho endothelial nitric oxide synthase (eNOS) (p-Ser1177), anti-phospho Akt and Akt antibodies from Cell Signaling Technology (Beverly, MA, USA); anti-eNOS antibody from BD Biosciences (San Jose, CA, USA).

2.7. Statistical analysis

All data are presented as mean ± standard error of the mean. Differences among groups were compared by one-way analysis of variance followed by the Tukey–Kramer method, using StatView software (SAS Institute, Inc., Cary, NC, USA). Student’s t test was used to assess differences between two groups when appropriate. The differences were considered statistically significant at a value of p < 0.05.
3. Results

3.1. CO₂ mist treatment attenuates infarct size

To demonstrate the biological functions of CO₂ mist in hearts, we first examined the effects of CO₂ gas or CO₂ mist on the infarct size in rat hearts (Fig. 1). Pretreatment with CO₂ mist significantly reduced the infarct size at 48 h after the MI induction, compared with no treatment. In contrast, pretreatment with only CO₂ gas did not affect the infarct size.

3.2. Effects of CO₂ mist pretreatment on body weight, hemodynamic status, heart weight and oxidative stress

At 3 days after MI induction, there were no significant differences among the groups in blood pressure and heart rate (Fig. 2A and B). Thus, pretreatment with CO₂ gas or CO₂ mist had no influence on the hemodynamic status. Nor was there any significant difference in body weight among the groups (Fig. 2C). At the same time point, the ratio of ventricular weight to body weight, which was increased by MI induction, did not differ between the CM group and the UT group (Fig. 2D).

Oxidative stress is known to involve remodeling of organs or tissue (12,17). MI-induced elevation of d-ROMs may contribute to a worsening of cardiac remodeling. Therefore, we measured the serum level of d-ROMs as the systemic oxidative stress, and the myocardial 8-OHdG content as the local oxidative stress. Pretreatment with CO₂ mist reduced the elevation of serum d-ROM levels (Fig. 2E). In contrast, pretreatment with CO₂ gas failed to reduce it. LV 8-OHdG content was significantly elevated by MI induction.
Pretreatment with CO2 mist significantly attenuated the myocardial 8-OHdG content after MI (Fig. 2F). Pretreatment with CO2 gas also attenuated it, although its effect tended to be weaker than pretreatment of CO2 mist.

3.3. Effect of CO2 mist on gene expressions in the marginal area of the infarction

The mRNA expressions in the LV marginal area in each group of rats were measured by quantitative real-time RT-PCR, 3 days after MI induction. The MI-induced increase in mRNA expressions of monocyte chemotactant protein-1 (MCP-1), tumor necrosis factor (TNF)-α, and matrix metalloproteinase-9 (MMP-9), factors closely related to cardiac inflammation, was significantly lower in the CM group but not in the CG group (Fig. 3A, B, and C).

The mRNA expressions of the collagens type I and III (Col-I and Col-III), and transforming growth factor-β1 (TGF-β1), which are factors closely associated with cardiac fibrosis, were remarkably increased by MI induction (Fig. 3D, E, and F). There was significantly less upregulation of Col-I and TGF-β1 in the CM group. Pretreatment with CO2 gas also decreased MI-induced TGF-β1 expression. Gene expressions of atrial natriuretic peptide and brain natriuretic peptides in the marginal area were similar among the groups (data not shown).

3.4. CO2 mist pretreatment prevents deterioration of LV function

Light micrographs of a cross-section of the mid-portion of the left ventricle at 7 days after MI induction (Fig. 4A) revealed that the LV size was smaller in the CM group than in the UT group. Although body weights in the MI groups were similar, the infarct size and ventricular weight were significantly lower in the CM group than in the UT group (Fig. 4B, C and D).

On echocardiographic evaluation 7 days after MI induction, LV end-systolic dimension, LVEDV, and LDESV were significantly smaller, whereas LVEF was significantly greater in the CM group compared with the UT group (Fig. 4E and Table 1). There was no difference in diastolic function, including E/A, among the groups.

3.5. Effect of CO2 mist on protein levels in the left ventricle

In order to elucidate the mechanism of the effect of CO2 mist in this model, certain protein levels were examined by western blot analysis (Fig. 5). Interestingly, treatment with CO2 mist for 7 days increased the phosphorylated eNOS and Akt levels in the rat left ventricle, compared with those of untreated rats. Furthermore, the Hsp72 level in the left ventricle of the CO2 mist-treated rats was significantly elevated, compared with that of untreated rats.

4. Discussion

The major finding of the present study is that pretreatment with CO2 mist reduced cardiac injury in rat hearts. Furthermore, we provided the first evidence that CO2 mist treatment increases the level of LV eNOS and Akt phosphorylations and Hsp72 in this model. As a result, CO2 mist treatment can reduce the infarct size.

Natural springs have been used as spa therapy in European countries for many years in order to treat a variety of diseases. It is
known that CO2-enriched water induces peripheral vasodilatation, which increases cutaneous blood flow (6–8). A recent study demonstrated that the immersion of a unilateral ischemic hind limb in CO2-enriched water causes an NO-dependent increase in collateral blood perfusion in mice (18). Furthermore, we have recently demonstrated that CO2 mist produced by a simple device, rather than a CO2 bath, could improve the development of cardiac remodeling after MI and accelerate angiogenesis in a mouse model of peripheral arterial disease (9,10). However, it was still unclear whether pretreatment with CO2 mist could protect cardiomyocytes against ischemic injury. Our present data suggest that pretreatment with CO2 mist, like remote ischemic preconditioning, has a cardioprotective effect in ischemic heart diseases (3,4).

Oxidative stress is another important factor that influences cardiac remodeling (17). The serum level of d-ROMs was increased by MI. Interestingly, pretreatment with CO2 mist led to significantly lower d-ROM levels (Fig. 2E). Furthermore, pretreatment with CO2 mist significantly attenuated MI-induced LV 8-OHdG content (Fig. 2F), indicating that this therapy could decrease both systemic and local oxidative stresses via probably the compensatory actions of some antioxidative systems, resulting in a reduction of the accumulation of inflammatory cells. However, the precise mechanism behind these results is still unclear.

The mRNA expression of MCP-1, TNF-α and MMP-9, which are inflammatory markers, was suppressed in the marginal area of the MI rats that were pretreated with CO2 mist. Moreover, pretreatment with CO2 mist suppressed the mRNA expression of Col-I and TGF-β1, which are markers of fibrosis. TGF-β1, in particular, is a pleiotropic cytokine that has been implicated as a major contributor to tissue fibrosis in various organ systems. Previous studies have shown that the expression of TGF-β1 mRNA is increased in the LV myocardium of patients with idiopathic hypertrophic cardiomyopathy or dilated cardiomyopathy, and in animal models of hypertension, myocardial infarction, and pressure overload (19,20). Thus, TGF-β1 may be responsible for the development of LV remodeling, and blocking of TGF-β1 pathways might be a pharmacological target for the prevention of cardiac remodeling. The results of this study suggest that the attenuation of these expressions by pretreatment with CO2 mist might salvage the ischemic myocardium and prevent increased LV enlargement in MI model rats.

A previous study has demonstrated the possible mechanisms of ischemic preconditioning by proteomic analysis using animal models, but it is still unclear which were the key proteins (21). To further investigate the mechanisms of the effect of CO2 mist, we finally compared the LV protein levels in rats treated with CO2 for 7 days with those of untreated rats. NO is known to be a key vaso-dilatory molecule that not only regulates vascular tone, but also has antiplatelet, anti-inflammatory, and antioxidant properties (22). NO production is also closely associated with the activation of eNOS, which has been used as an index of vascular endothelial cell function (23). A lack of eNOS has been implicated in accelerated vascular remodeling with high-flow stress (24). The eNOS-dependent NO production promotes angiogenesis and suppresses cardiac fibrosis in the repair process of pressure-overloaded cardiac dysfunction (25,26). Interestingly, treatment with CO2 mist...
increased its activation, suggesting that increased eNOS activity may result in improved LV function in this model.

Hsps are expressed in both prokaryotes and eukaryotes, and are generally highly conserved. Hsp72, which is the most prominent member of the Hsp70 family, is located within both the cytoplasm and the nucleus. Numerous studies have shown that Hsp72 contributes to the prevention of protein aggregation, assists in refolding damaged proteins, chaperones nascent polypeptides along ribosomes, aids in the removal of damaged proteins, and is involved in protecting cells against a variety of stresses (27–29).

Notably, recent studies have reported that moderate exercise, combined with certain agents, can exert a cardioprotective effect by increasing Hsp72 protein levels in the heart and liver (30, 31). In the present study, treatment with CO2 mist significantly increased Hsp72 protein levels in the left ventricles of rats. Thus, increased Hsp72 protein levels may be a topic of great interest in the discussion as to how best to prevent MI-induced LV dysfunction. However, the mechanistic relation between CO2 mist and LV Hsp72 levels remains unclear. Further studies are needed to adequately explore the precise mechanisms underlying the effect of CO2 mist on LV function.

4.1. Study limitation

Our previous study have shown that there was no effect of arterial and venous blood gases with CO2 mist and CO2 gas treatment under either chronic or acute conditions (9). In contrast, the tissue saturation levels were significantly increased in the CO2 mist treatment group compared with the CO2 gas treatment group, suggesting that CO2 mist had increased blood flow effects compared with CO2 gas alone. It could result in shortened blood capillary transit time, which would cause a reduction in the extraction of oxygen from the blood and release oxygen more easily. However, the observations in the present study do not show the precise mechanism of cardioprotective effects of CO2 mist. It is also unclear whether repetitive treatment of CO2 mist has an effect on other organs such as blood vessels and kidney. Therefore, further studies are needed to elucidate the precise mechanism of the effects of CO2 mist before clinical applications.
In conclusion, we have demonstrated that pretreatment with CO₂ mist may have an ischemic preconditioning effect by increasing eNOS activation and Hsp72 levels while suppressing oxidative stress, resulting in the attenuation of infarct size. CO₂ mist preparative treatment might be potentially useful for preserving myocardium in ischemic heart diseases.

Conflict of interest

None declared.

Funding

This study was supported in part by Grant-in-Aid for Scientific Research (24591101 and 26460344) from the Ministry of Education, Culture, Sports, Science and Technology, ACP JAPAN Co., Ltd., and Hoansha Foundation.

Acknowledgments

The authors thank Ms. Chiori Asahi for technical assistance.

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