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Cardiac Effects of Acute Administration of a Protonophore in a Rat Model

Running Head: Protonophore and the Heart

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Abstract

Introduction: Excessive use of uncoupling agents, previously used as weight-loss agents, has led to the increase of body temperature and death. The aim of the present study was to evaluate the acute cardiac effects of mitochondrial protonophore in a rat model at a high dose, and its specific influence on cardiac substrate uptake.

Methods*:* Eight-week-old male Sprague-Dawley rats were intraperitoneally injected with the protonophore carbonyl cyanide m-chloro phenyl hydrazone (CCCP; 4 mg/kg) or vehicle (dimethyl sulfoxide). Blood pressure, heart rate (HR), and systolic function was recorded. Substrate uptake was monitored by radio-active tracers.

Key findings: Compared to the control group, the respiratory rate and body temperature increased, the left ventricle was dilated, and systolic function transiently deteriorated in the CCCP group. There was no difference in blood pressure and heart rate between the two groups. In cardiac substrate uptake, glucose uptake showed a 95% increase ($p < 0.05$), and fatty acid uptake showed a 52% decrease ($p < 0.05$) in CCCP-administered group.

Conclusion: The deleterious effects on cardiac function and the changes in substrate uptake were observed when administered with the protonophore at a high dose.

Key words: cardiac function; protonophore; substrate uptake.

Introduction

Mitochondria are crucial modulators of viability and death in a variety of cell types, and play important roles in energy production [1-2]. In brown fat cells, mitochondrial respiration is uncoupled from ATP synthesis and heat is produced instead that the energy from beta-oxidation is converted into ATP [3]. Uncoupling also occurs to some extent in other cell types. Since the 1940s, several substances including carbonyl cyanide m-chloro phenyl hydrazone (CCCP) and 2,4-dinitrophenol have been known to act as uncoupling agents [5,6]. These uncoupling agents have the nature of lipid-soluble acids and provide a bypass pathway of H^+ across the inner mitochondrial membrane as a protonophore. As a result of this short cut, the proton-motive force is dissipated and ATP cannot be synthesized. Recently, several lines of evidence showed that at low doses, uncouplers reduced reactive oxygen species (ROS) [4,7,8], increased energy expenditure [4,7], and improved longevity [4,8].

Historically, uncoupling agents were used as so-called “weight-loss agents” [9], and their excessive use due to psychological problems has led to the increase of core body temperature and even death [10]. These weight-loss agents are also associated with a danger of overuse due to the image of so-called ideal beauty. The toxic effects of protonophoric mitochondrial uncouplers have been extensively described [10], and the marked toxicity had motivated its withdrawal from the market. However, recent evidences of long administration of chemical

uncoupling [4,7,8] make the agent began to show sings coming life again, for example, as a patent of the new derivatives for the use of non-alcoholic fatty liver disease. However, detailed knowledge of the effects of their acute administration at a high dose to the heart, which is one of most energy-consuming organs, have not been examined.

We previously reported the uptake of the radioisotope-labeled tracer technetium 99m Technetium (^{99m}Tc)-sestamibi (MIBI) signals in the perfused and excised heart in a rat model administered a protonophore, CCCP [11]. In the excised hearts of Sprague-Dawley (SD) rats administered ^{99m}Tc-MIBI which is positively charged and distributed into mitochondria according to the mitochondrial membrane potentials, CCCP decreased the ^{99m}Tc-MIBI signals along with a decrease of *in situ* ATP and phosphocreatine contents of the heart. In the present study, we aimed to clarify the acute effects of the protonophore on cardiac function and cardiac substrate uptake in a rat model, which are the novel points of the present study.

Methods

Animals and materials

Eight-week-old male SD rats (body weight 280–290 g) were administered CCCP (Wako Pure Chemical Industries; Osaka, Japan). Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University (permission no. MedKyo14184) and conducted following the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health. ^{99m}Tc-MIBI and ^{125}I-(p-iodophenyl)-9-R,S-methylpentadecanoic acid (9MPA) were purchased from FUJIFILM RI Pharma Co. Ltd. (Tokyo, Japan). ^{18}F-deoxyglucose (FDG) was synthesized by Kyoto University Hospital. CCCP (Wako Pure Chemical Industries, Osaka, Japan) was diluted in 100% dimethyl sulfoxide (DMSO, Wako Pure Chemical Industry, Osaka, Japan) to prepare a 10 mM stock solution.

Physiological and hemodynamic analysis

Protocol 1: To investigate the physiological and hemodynamic changes in CCCP-administered rats, 8-week-old male SD rats (n = 6) were intraperitoneally injected with CCCP (4 mg/kg) or vehicle (DMSO; n = 6). Body temperature and blood pressure was recorded at the rectum at 30 min after the CCCP injection (AD-1687, A&D Company Ltd., Tokyo, Japan). Blood pressure is determined by the tail-cuff method using a noninvasive automated blood

pressure apparatus (Softron SBP-200, Softron Co. Ltd., Tokyo, Japan) without anesthesia. Transthoracic echocardiographic analysis was performed as previously reported [12] using a Sonos-5500 echocardiograph (Agilent Technologies, Santa Clara, CA, USA) with a 15-MHz linear transducer. Heart rate (HR), intraventricular septal thickness (IVSd), left ventricular dimension in the diastolic phase (LVDd), and left ventricular dimension in the systolic phase (LVDs) were measured with M-mode echocardiography 30 min, 90 min, and 180 min after CCCP injection, and fractional shortening (FS) was calculated using the following formula: %FS = [(LVDd –LVDs)/LVDd] × 100.

Effect of CCCP on the uptake of a glucose and fatty acid radiotracer

Protocol 2: To analyze the effect CCCP on glucose and fatty acid uptake, ^{18}F-deoxyglucose (FDG) and ^{125}I-9MPA was used, respectively. The rats (n = 6 per group) were fasted overnight, administered CCCP, and injected with 1 mCi of ^{18}FDG and 20 μCi of ^{125}I-9MPA simultaneously 45 min later. They were euthanized by decapitation 45 min after the injection, and the hearts were removed and washed in cold saline. The 1/3 portion of the apical side was frozen in liquid nitrogen and the radioisotopic activity was measured using a scintillation counter (Cobra2^{TM} Auto-gamma, Packard) [13, 14]. To measure ^{18}FDG uptake, radioisotopic activity was measured just after euthanization because the half-decay time of ^{18}FDG is 110 min. To measure ^{125}I-9MPA uptake, radioisotopic activity was measured 48 h after the

euthanization. The myocardial uptake levels of ^{18}FDG or ^{125}I-9MPA were assessed by direct measurement using the scintillation counter. The amount of radioisotope incorporated is expressed as a percentage of the administered radioisotope activity corrected by heart weight (g). Cross-talk between the two tracers was negligible [13, 14].

Washout of ^{99m}Tc-MIBI *in vivo*

Protocol 3: In order to investigate the mechanism of substrate change, we calculated ^{99m}Tc-MIBI *in vivo* to show the changes in mitochondrial function [15, 16, 17]. A dose of 15 MBq (405.4 μCi) of ^{99m}Tc-MIBI was injected into the tail vein under anesthesia with pentobarbital sodium (10 mg/kg IP). Rats were placed exactly 10 cm from the collimator. Pre-CCCP or vehicle-administered images (64 × 64 matrix size) were obtained 15 min after the ^{99m}Tc-MIBI injection. Then, CCCP or vehicle was administered intraperitoneally to rats 90 min after the ^{99m}Tc-MIBI injection (CCCP: n = 8, vehicle: n = 7). Thereafter, post-CCCP or vehicle-administered images were obtained 180 min after the ^{99m}Tc-MIBI injection. To calculate the rate of myocardial ^{99m}Tc-MIBI washout following injection, a region of interest was manually drawn around the heart and in the mediastinum area between the upper limbs. The myocardial ^{99m}Tc-MIBI washout rate (percentage) was calculated using the following equation: (A – B*DC) / A × 100 (%), in which A was defined as (pre-CCCP or vehicle-administered heart count − pre-CCCP or vehicle-administered mediastinum count), B was defined as (post-CCCP

or vehicle-administered heart count − post-CCCP or vehicle-administered mediastinum count), and DC is the decay coefficient. [15, 16, 17]

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Differences between the groups were compared using the Kruskal-Wallis post-hoc using Dunn's test. In all tests, a value of $p < 0.05$ was considered statistically significant.

Results

Effects of CCCP on body temperature, hemodynamics, and cardiac function

Body temperature analyzed at 30 min after the CCCP injection was higher than that after vehicle injection ($p = 0.024$, Figure 1A), indicating that electron transport was uncoupled by CCCP. There was no difference in heart rate between CCCP-administered and vehicle-administered rats (Figure 1B). Blood pressure tended to decrease in CCCP-administered rats compared to vehicle-administered rats ($p = 0.086$, Figure 1C). Serial echocardiographic examination showed that both LVDd and LVDs increased ($p = 0.0048$ and 0.0047, respectively) and fractional shortening decreased ($p = 0.0078$) at 30 min after the CCCP injection (Figures 1D, 1E, and 1F, respectively), indicating that CCCP caused transient left LV dilatation and systolic dysfunction. The difference between CCCP-administered and vehicle-administered rats was diminished at 90–180 min after the CCCP injection.

CCCP changed substrate uptake in the cardiac tissue

Next, we examined whether CCCP caused a change in the myocardial uptake of glucose and fatty acids using ^{18}FDG and ^{125}I-9MPA, respectively (Figure 2A). Compared to the vehicle group, glucose uptake showed a 95% increase ($p = 0.033$) and the fatty acid uptake showed a 52% decrease ($p = 0.033$) 90 min after CCCP administration (Figures 2B and 2C, respectively), indicating that the protonphore caused changes in substrate uptake.

149

150 **^{99m}Tc-MIBI washout increased in rats administered CCCP**

151 To investigate the effect of CCCP on membrane potentials *in vivo*, we obtained pre-CCCP or

152 vehicle-administered images 15 min after the ^{99m}Tc-MIBI injection, then CCCP or vehicle

153 was injected, and post-CCCP or vehicle-administered images were obtained 180 min after the

154 ^{99m}Tc-MIBI injection (Figure 3A). Myocardial retention of ^{99m}Tc-MIBI was markedly

155 decreased after the CCCP injection (Figure 3B, lower panels) compared to that in vehicle-

156 administered rats (Figure 3B, upper panels). The analysis of *in vivo* images showed that the

157 washout rate of ^{99m}Tc-MIBI was significantly increased in CCCP rats ($p = 0.015$; Figure 3C).

158

Discussion

In summary, CCCP caused transient LV dilatation and systolic dysfunction. CCCP increased glucose uptake, and decreased fatty acid uptake in the rat heart tissue and ^{99m}Tc-MIBI washout rate *in vivo*.

We recently reported that the accumulation of ^{99m}Tc-MIBI signals was correlated to the tetramethylrhodamine ethyl ester assay in *ex vivo* perfused rat hearts [11]. We found that CCCP decreased the *in situ* ATP levels at 30 min after the injection [11], suggesting that energy deficiency might cause the LV dilatation and systolic dysfunction observed in the present study. This mechanism of the cardiac dysfunction is currently only speculative and was not directly elucidated in the present study; however, the effects of CCCP were transient according to the metabolic rate of CCCP. Dillis et al. [18] reported that hepatic ATP and co-substrate levels decreased 30 min after CCCP injection and returned to normal at 60 min after the injection, which is consistent with the results of the present study. ^{9m}Tc-MIBI has a high affinity for the negative charges associated with membrane potentials across the mitochondrial membrane, according to the Nernstian equation [19,20]. A blood clearance study showed that myocellular equilibrium was reached at a $t_{1/2}$ of 2–5 min in clinical use [21]. Therefore, the washout rate was increased according to the decreased membrane potentials. The observed increase in ^{99m}Tc-MIBI washout rate in the present study has the

178 possibility to represent, at least partly, a decrease in mitochondrial membrane potentials and
179 dysfunction of mitochondria which support the CCCP-induced changes of the substrate
180 uptake (Figure 2) and energy deficiency [11] in the heart.
181
182 ^{18}FDG uptake increased in the present study. Although neither the metabolic rate of glycolysis
183 nor the molecular mechanism for directly increasing ^{123}FDG uptake was examined in the
184 present study, a possible mechanism of this rapid regulation is adenosine monophosphate
185 (AMP)-activated protein kinase. An increase of the AMP to ATP ratio, i.e. energy deficiency,
186 activated AMP-activated protein kinase and enhanced glucose uptake and glycolysis [22]. By
187 contrast, the uptake of ^{123}I-9MPA decreased. ^{123}I-9MPA was rapidly metabolized to
188 iodophenyl-3-methylnonanoic acid (3MNA) by beta-oxidation, and was not further
189 metabolized [23,24]; therefore, it is generally considered to reflect fatty acid oxidation in
190 mitochondria [23,24]. Ikawa et al. reported the increased iodine-123-labelled 15-(p-
191 iodophenyl)-3-(R,S)-methylpentadecanoic acid (^{123}I-BMIPP), another tracer of fatty acids, in
192 patients with mitochondrial cardiomyopathy with the increase in ^{99m}Tc-MIBI washout ratio
193 [25]. Most of the ^{123}I-BMIPP was incorporated into the triglyceride pool, and reflects the
194 turnover of the triglyceride pool in the cytosol [26]. In patients with mitochondrial
195 cardiomyopathy, the energy production shifts from the metabolism of fatty acids to the
196 glycolytic pathway with the excess of glycerol-3-phosphate, leading to the enhanced synthesis

of triglycerides. Thus, in chronic mitochondrial failure, ^{123}I-BMIPP is incorporated more into triglyceride-pool and remains in triglyceride pool in the cytosol [26]. Thus, decreased uptake of ^{123}I-9MPA reflects the acute mitochondrial dysfunction, and increased uptake (and decreased washout) of ^{123}I-BMIPP reflects the chronic mitochondrial dysfunction.

Life-long administration of low-dose chemical uncoupling 2,3-dinitrophenol to mice caused no adverse effects, decreased body weight, and prolonged survival [4][27]. However, the dose used in the present study caused LV dysfunction. One report showed that CCCP decreased hepatic ATP production when administered to rats at a dose of 4 mg/kg with no mortality, whereas a dose of 5 mg/kg resulted in 11% mortality [18]. The LD50 was found to be approximately 8 mg/kg. Hence, the dose of CCCP used in this short-term experiment is thought to be relatively high, indicating that high-dose CCCP is detrimental for cardiac function. The next key question is to determine to what dosage and for how long uncoupling would have to be increased to achieve beneficial effects due to decreased ROS production and to avoid detrimental effects due to decreased ATP production and heart failure.

Limitations

The limitations of the present study include the lack of an observed dose-response and the lack of a clear mechanism to explain the observed effects. Although the relationship between

^{99m}Tc-MIBI accumulation and mitochondrial potential was assessed in cultured myocytes [28], direct monitoring of the mitochondrial potential *in vivo* was also difficult to achieve; however, further studies about serial measurements of the phosphocreatine and βATP levels *in vivo* would provide useful information on the energy deficiency and its recovery in this model. Lack of measuring oxygen consumption rate in vivo is another limitation. Finally, we acknowledged that this work was a subsequent series of studies using CCCP [11], although the data in this work provided the insights on the changes in substrate uptake and function when we used CCCP.

Conclusions

The deleterious effects on cardiac function and the changes in substrate uptake were observed when administered with the protonophore at a high dose.

Competing interest

None declared.

Founding

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Acknowledgments

The founders had no the role in design, in the collection, analysis, and interpretation of data;

in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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301

Figure legends

Figure 1: Physiological and echocardiographic examination of CCCP-administered rats

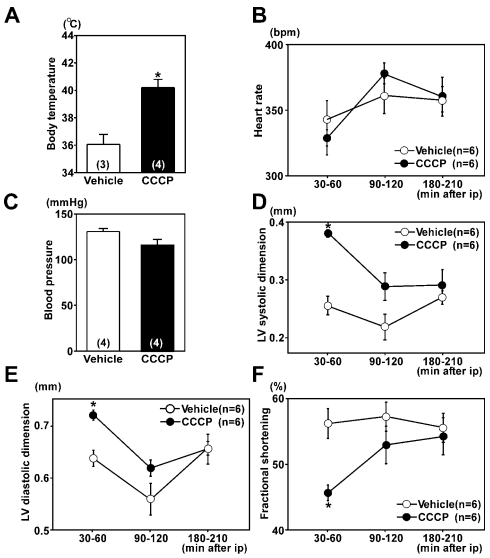
(A) Body temperature analyzed at 30 min after the CCCP injection was higher than that after vehicle injection. (B) Heart rate did not differ between CCCP-administered and vehicle-administered rats (Vehicle: n = 6, CCCP: n = 6). (C) Blood pressure tended to decrease in CCCP-administered rats (116 ±4 mmHg) compared to vehicle-administered rats (131 ± 3 mmHg). Vehicle: n = 6, CCCP: n = 6. (D) Left ventricular diastolic dimension (LVDd). Vehicle: n = 6, CCCP: n = 6. (E) Left ventricular systolic dimension (LVDs). (F) Fractional shortening (FS). Serial echocardiographic examination showed that both LVDs and LVDd increased and FS decreased up to 60 minutes after the CCCP injection. All circles and bars indicate means and SEMs respectively. *$p < 0.05$ versus vehicle-administered rats.

Figure 2: The uptake of ^{18}FDG was increased and the uptake of ^{125}I-9MPA was decreased by CCCP.

(A) A schema of the study for analyzing the extracted hearts. (B) and (C) The uptake of ^{18}FDG and ^{125}I-9MPA, respectively. All bars indicate means and SEMs. *$p < 0.05$ versus vehicle-administered rats. n=6 in each group.

Figure 3: ^{99m}Tc-MIBI washout was increased in rats administered CCCP

(A) A schema of the study for analyzing the images and extracted hearts. (B) Representative *in vivo* images of ^{99m}Tc-MIBI distribution. Myocardial retention of ^{99m}Tc-MIBI was markedly decreased after the CCCP injection (lower panels) compared to vehicle-administered rats (upper panels). White arrowheads indicate hearts. (C) Analysis of *in vivo* images showed that the ^{99m}Tc-MIBI washout rate was significantly increased in CCCP rats (Vehicle: n = 7, CCCP: n = 8). WR, washout rate. All bars indicate means and SEMs. *$p < 0.05$ versus vehicle-administered rats.



329

330 Figure 1

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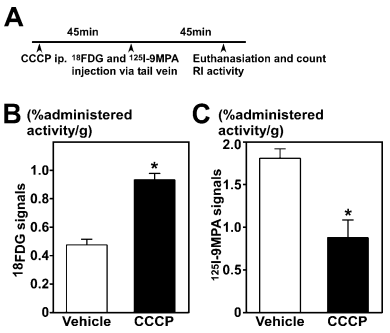
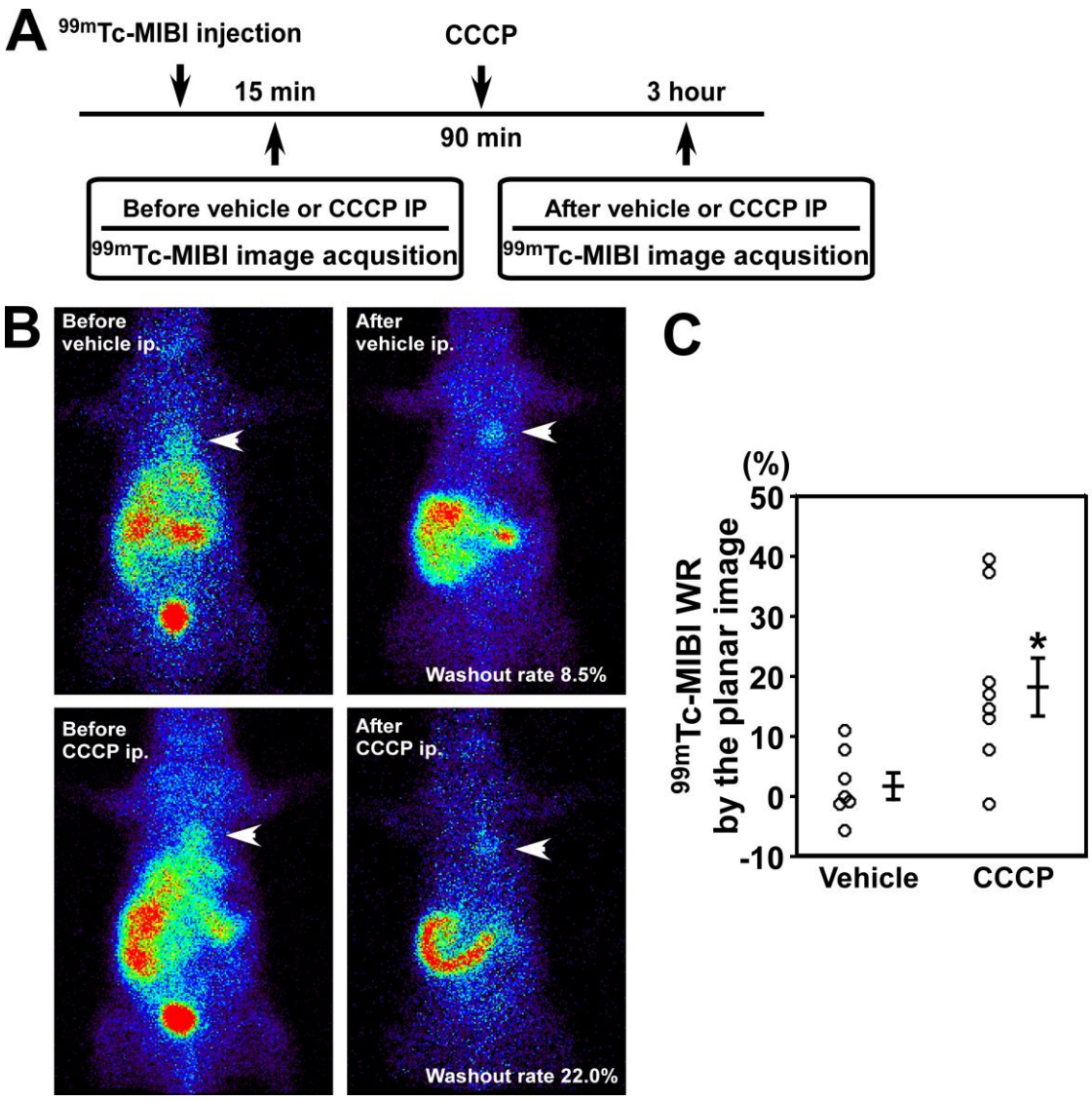

A
45min
45min
CCCP ip.
18FDG and 125I-9MPA injection via tail vein
Euthanasiation and count RI activity
B (%administered activity/g)
18FDG signals
1.0
0.8
0.6
0.4
0.2
0
Vehicle
CCCP
C (%administered activity/g)
125I-9MPA signals
2.0
1.5
1.0
0.5
0
Vehicle
CCCP

Figure 2



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336 Figure 3