Short Communication

Evaluation for effect of hypothermia on the disposition of 4-nitrophenol in rats by *in vitro* metabolism study and rat liver perfusion system

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RUNNING HEAD: Effect of hypothermia on the disposition of 4-nitrophenol

Abstract:

Objectives. The aim of this study was to evaluate the effect of hypothermia on the *in vivo* pharmacokinetics of 4-nitrophenol (4NP) using rat liver homogenate and rat liver perfusion system.

Methods. Rat liver homogenate was incubated with 4NP, which is mainly metabolized by CYP2E1, at 37, 34, 32, or 28°C. The Michaelis constant (K_m) and maximum elimination velocity (V_{max}) of 4NP were calculated by a Hanes-Woolf plot. The hepatic extraction ratio (E_h) of 4NP was evaluated in a rat liver perfusion study at 37, 34, 32, or 28°C. Moreover, the plasma concentration profiles of 4NP after its i.v. administration to rats were analyzed by the moment theory and were compared to *in vitro* parameters.

Key findings. While the K_m of 4NP was not changed, the V_{max} and E_h were reduced at low temperatures. The plasma concentrations of 4NP after its i.v. administration to rats were significantly increased at 28°C.

Conclusion. Changes in the pharmacokinetics of 4NP under hypothermic conditions were caused by alterations in V_{max} and E_h. We may be able to predict the disposition of a drug by *in vitro* studies.

KEY WORDS: 4-nitrophenol, hypothermia, CYP2E1, liver perfusion

Introduction

Therapeutic hypothermia is beneficial for patients with acute myocardial infarction or post cardiac arrest syndrome [1-5]. Several drugs such as propofol, midazolam, or dexmedetomidine have been used during hypothermia to cause a sedative action or negate the complications of hypothermia [5, 6]. However, the pharmacokinetics of drugs used during therapeutic hypothermia have been shown to be altered [7-9]. We need to identify the factors affecting the disposition of a drug to optimize medication. We previously reported that the pharmacokinetics of phenolsulfonphthalein (PSP), indocyanine green (ICG), and fluorescein isothiocyanate-dextran (FD-4, MW 4400) as marker compounds and their hepatic disposition under hypothermic conditions in rats could differ with the disposition route and intrinsic clearance of these drugs [10, 11]. Despite of necessity to determine the individual factors affecting drug disposition for prediction the pharmacokinetics during hypothermia, it has not been clarified. In this study, we tried to evaluate the effect of temperature on drug disposition by focusing on the hepatic disposition.

In this study, we chose 4-nitrophenol (4NP) as a model compound metabolized in the liver by CYP2E1 [12, 13]. We thought we could evaluate the effect of hypothermia on a drug disposition metabolized by CYP2E1 which plays a major role in the metabolism

of several drugs used during hypothermia such as acetaminophen, isoflurane, isoniazid, and theophylline [14-18].

In the present study, we examined the effect of low temperature on the CYP2E1 activity and hepatic extraction ratio of 4NP. Moreover, we evaluated the relationship between the *in vitro* and *in vivo* pharmacokinetic parameters of 4NP to evaluate the possibility of predicting changes in the pharmacokinetics of drugs under hypothermic conditions by an *in vitro* study.

Materials and Methods

Materials

4NP was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All chemicals were of the highest purity available.

Animals

Male Wistar rats (180-210 g or 240-270 g) were housed in a cage in an air-conditioned room and maintained on a standard laboratory diet (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments in the present study conformed to the Guidelines for Animal Experimentation of Nagasaki University and

were approved by the Committee of Animal Experimentation of Nagasaki University (Approval number: 0506280443).

Liver homogenate study

The liver was removed from male Wistar rats (180-210 g) and homogenized in cold Tris/HCl buffer containing 5 mM $MgSO_4$ (pH 7.4). Rat liver homogenate was incubated at 37, 34, 32, or 28°C for 15 min after the addition of 4NP (50, 100, 200, 400, 800 μg/mL) and several lots of the liver homogenate were used in this study. We have preliminary examined the elimination of 4NP from liver homogenate until 15 min followed the first elimination manner (data not shown). After incubation, the incubation mixture was mixed with acetone to stop the metabolism reaction and centrifuged for 5 min at 15,000 rpm. The remaining concentration of 4NP was determined by spectrophotometer and then the eliminate velocity of 4NP was calculated by Eq.1

$$v = \frac{(C_0 - C_{15}) \times V}{15} \div mg\ protein \quad (1)$$

where v is the eliminate velocity, C_0 and C_{15} represent the concentration of 4NP at 0 and 15 min, respectively, and V is the incubation volume (1 mL).

The Michaelis constant (Km) and maximum eliminate velocity (Vmax) were

calculated by a Hanes-Woolf plot (Eq.2).

$$\frac{C}{v} = \frac{1}{V_{max}} \times C + \frac{K_m}{V_{max}} \quad (2)$$

where v is the eliminate velocity normalized by protein content of the homogenate, C is the concentration of 4NP, K_m is the Michaelis constant, and V_{max} is the maximum eliminate velocity.

Liver perfusion study

Male Wistar rat liver was perfused *in situ* as described previously [11]. After a stabilization period of 30 min, the 4NP solution (20 mg/mL x 0.1 mL) was injected into the perfusion route. After administration of the 4NP solution, venous outflow samples were collected into tubes for 5 min. The hepatic extraction ratio (E_h) was calculated as follows Eq 3 with the assumption that the hepatic disposition of 4NP was allowed to well-stirred model.

$$E_h = \frac{D - C_{out} \times V_{out}}{D} \quad (3)$$

where D is administration dose of 4NP, C_{out} is concentration of 4NP in outflow effluent and V_{out} is the volume of outflow effluent.

In vivo study

Male Wistar rats (240-270 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the left femoral artery was cannulated with a polyethylene tube (i.d. 0.25 mm, o.d. 0.61 mm, Dual Plastics, Dural, Australia).

Rats were divided into four groups: a control group in which rectal temperature was maintained at 37^{o}C by a heat lamp throughout the procedure; a hypothermic group kept at 34^{o}C, 32^{o}C, or 28^{o}C, in which hypothermia was induced by external cooling with icepack on their body before the administration of the drug, and rectal temperature was maintained at 34, 32, or 28^{o}C.

The drug solution (20 mg/mL x 0.1 mL) was injected into the right femoral vein. After administration of the drug solution, blood was collected at the selected times from the heparinized cannula inserted into the femoral artery until 50 min. Blood was centrifuged at 15000 rpm for 5 min.

Moment parameters (AUC_p and MRT_p) were calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation [19].

121 Assay

122 The concentration of 4NP was determined spectrophotometrically at 410 nm
123 after dilution with 1 M NaOH [20].

124

125 Statistical analysis

126 Statistical comparisons were performed by Dunnett's test after examining with
127 an analysis of variance (ANOVA) or repeated measured ANOVA. $P < 0.05$ was
128 considered to be indicative of significance compared to control group (37°C). The
129 results were expressed the mean ± S.E.

Results

Effect of temperature on 4NP metabolism in rat liver homogenate

The Michaelis constant (K_m) and maximum eliminate velocity (V_{max}) of 4NP at 37, 34, 32, and 28°C were obtained from the Hanes-Woolf plot (fig.1A). The V_{max} of 4NP was decreased to about 30% at 32°C and 70% at 28°C compared to 37°C and there was a significant difference between 37°C and 28°C. (Fig 1B) The Km of 4NP was not altered at 34°C and 32°C compared to 37oC. (Fig 1B) The Km at 28°C was decreased by the half from 37°C although not significant.

Change in the hepatic extraction ratio at low temperatures in the rat liver perfusion system

Fig.2 illustrates the E_h of 4NP obtained by the rat liver perfusion study at each temperature. The E_h at 28°C was decreased about 15% compared to 37°C although the difference was not significant. The E_h of 4NP was linearly decreased according to the reduction of temperature (r^2=0.931, p=0.034).

Pharmacokinetics of 4NP *in vivo* in rats under hypothermic conditions

Fig.3 shows the plasma concentration – time profile of 4NP after its i.v. administration to rats under different body temperatures. The plasma concentration of 4NP at 28°C was significantly higher than that at 37°C. The AUC_p, MRT_p, and CL_{tot} of

149 4NP at each temperature are listed in Table I. The AUC_p of 4NP was 1.7 (34°C), 2.9
150 (32°C), and 5.5 (28°C) times greater than that at 37°C, and the MRT_p of 4NP was
151 significantly prolonged at 32 and 28°C. In addition, the CL_{tot} of 4NP was significantly
152 lower at 32 and 28°C than that at 37°C.
153
154 Discussion
155 We performed an *in vitro* metabolism study using rat liver homogenate and
156 isolated liver perfusion study to evaluate the effect of temperature on the elimination in
157 the liver homogenate and E_h of 4NP. We evaluated the effect of temperature on 4NP
158 metabolism activity in rat liver homogenate since liver homogenate containing the
159 metabolic enzymes or co-enzymes necessary to metabolize drugs and easy to handling
160 compared to another method. The V_{max} of 4NP decreased according to the temperature,
161 while no significant difference was observed in K_m. This suggests that the affinity of
162 4NP with CYP2E1 was not affected by temperature, whereas the eliminate velocity could
163 have been altered under hypothermic conditions. Similar to our result, it has been
164 reported that the Vmax of midazolam metabolized by CYP3A4 was decreased at 33°C
165 compared to 37°C while the Km was not altered [21]. The previous study [18] has shown
166 that NADPH, NADPH-cytochrome P-450 reductase, and lipids are required for

metabolism by CYP. These factors also produced by enzymatic reaction and the activity of these enzymes could also be decreased under hypothermia. Further study is needed to clarify the mechanisms the change in Vmax under hypothermic condition.

Moreover, we performed an isolated liver perfusion study to analyze changes in the E_h of 4NP at low temperatures. The isolated liver perfusion study is a useful method to evaluate the effect of temperature on the hepatic uptake of 4NP because we can easily control the flow rate and perfusion temperature. Since E_h is affected by these factors, we ran the liver perfusion study under a constant flow rate and protein-free conditions.

The E_h of 4NP at 37^{o}C was approximately-same as the reported value obtained under the steady-state condition [22] and it was decreased according to temperature. E_h is influenced by the liver blood flow rate, protein binding ratio, and hepatic intrinsic clearance ($CL_{int,h}$). The reduction in E_h could have been caused by alterations in $CL_{int,h}$, owing to the constant flow rate and protein-free conditions. $CL_{int,h}$ is divided into several processes including influx into the cell or efflux from the cell and metabolism by the enzymes in the cell. The uptake process of 4NP into the liver has not been fully identified. Quebbeman has reported that the organic anion transporter (Oat) is related to 4NP uptake into the liver [23].

Concerning Oat activity under hypothermic conditions, a decrease in the uptake

185 of phenolsulfonphthalein into the liver via Oat was suggested in our previous study [11].
186 Furthermore, we showed a reduction in CYP2E1 activity at low temperatures in this study,
187 and this alteration may have also had an effect on the E_h of 4NP. Thus, the reduction in
188 CYP2E1 and Oat activity could cause changes in the E_h of 4NP under hypothermic
189 conditions.

190 As the next step, we evaluated the pharmacokinetics of 4NP in rats to identify
191 how alterations in the hepatic disposition affected the pharmacokinetics of 4NP in rats.
192 As illustrated in Fig.2, the plasma concentration of 4NP was significantly increased at
193 28^{o}C and the CL_{tot} of 4NP was decreased according to a reduction in the body temperature
194 (Table I). In general, the hepatic clearance of a drug depends on hepatic blood flow and
195 E_h. It has been reported that the blood flow was reduced under hypothermia[24].
196 Moreover, we determined the protein binding ratio of 4NP with BSA by equilibrium
197 dialysis method and it was slightly increased at 28^{o}C compared to 37^{o}C (data not shown).
198 These results suggest that the reduction of CL_{tot} might be caused by reduction of E_h,
199 hepatic blood flow or unbound fraction of 4NP.

200 Conclusion

201 We showed that the elimination velocity from homogenate and E_h of 4NP were
202 decreased under hypothermic conditions and that these alterations could affect the

pharmacokinetics of a drug under hypothermic conditions in rats. These results may be helpful in predicting the pharmacokinetics of a drug during hypothermia.

Acknowledgement

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209 Table

210 Table I Pharmacokinetic parameters for the plasma concentration time profiles of 4NP

211 after its i.v. administration to rats at a dose of 2 mg under different temperatures.

Body temperature (^oC)	37	34	32	28
AUC_p (μg･mL/min)	106.4 ±16.9	207.5 ±111.1	372.7 ±42.2	727.0** ±140.5
MRT_p (min)	6.5 ±1.1	15.5 ±5.3	34.0** ±4.7	48.4** ±5.2
CL_{tot} (mL/min)	20.7 ±3.1	17.9 ±5.7	5.7** ±0.7	3.2** ±0.6

212 AUC_p: area under the plasma concentration-time profile, MRT_p: mean resistance time,

213 CL_{tot}: total body clearance.

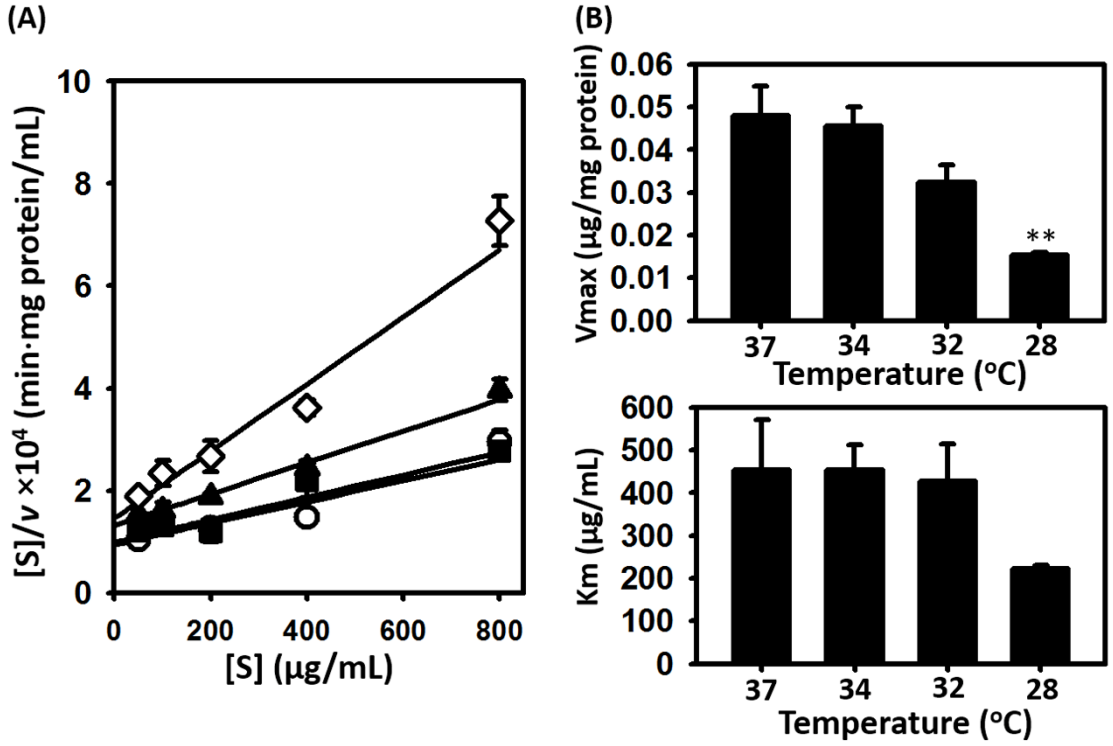
214 The AUC_p, MRT_p, and CL_{tot} represent the mean ± S.E. of at least four experiments.

215 ** $p < 0.01$: significantly different from the result at 37^oC

216

Legend to Figures

Fig.1 (A) Hanes-Woolf plots of 4NP elimination from rat liver homogenate under different temperatures and (B) Km and Vmax of 4NP at each temperature obtained by Hanes-Woolf plots. Each point represents the mean ± S.E. and bar represents the mean + S.E. of at least three experiments. Key: at 37°C (○), 34°C (■), 32°C (▲), or 28°C (◇). **: p<0.01, significantly different from 37°C.

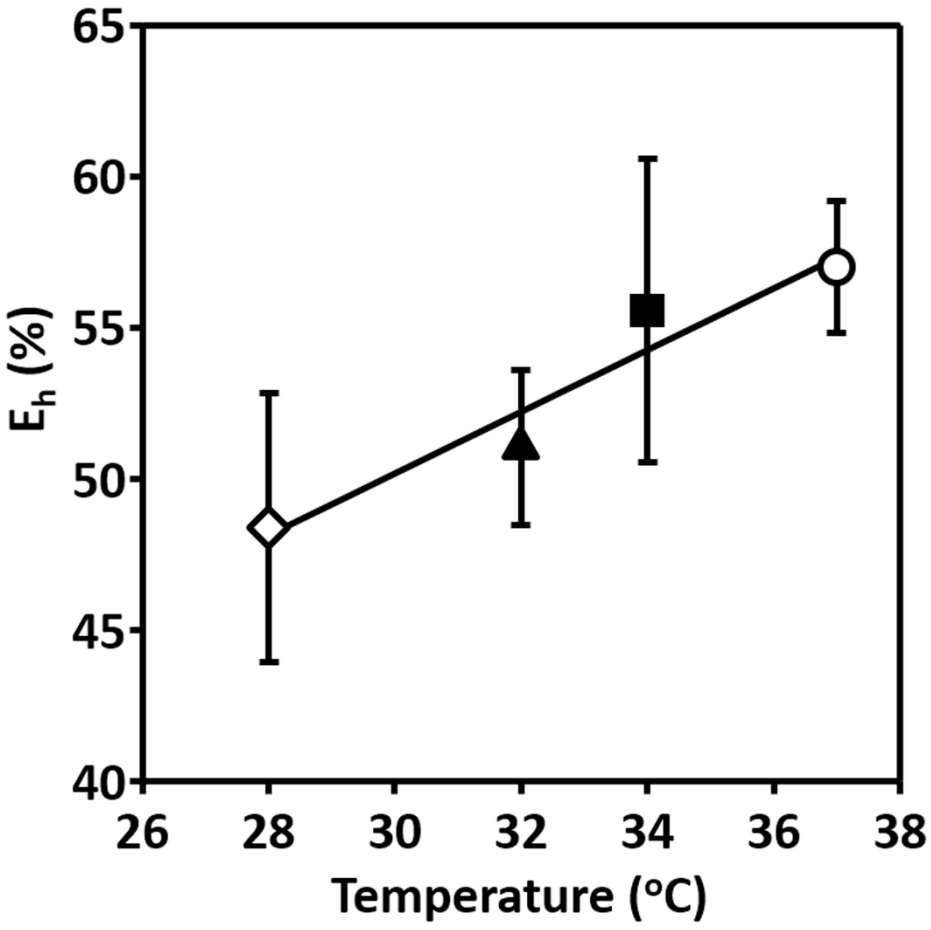


225 Fig.2 Relationship between the perfusate temperature and hepatic extraction ratio (E_h)

226 of 4NP at a dose of 0.2 mg in the rat liver perfusion system. Each symbol represents the

227 mean ± S.E. of at least five experiments. The solid line represents the regression curve.

228 Key: at 37°C (○), 34°C (■), 32°C (▲), or 28°C (◇).



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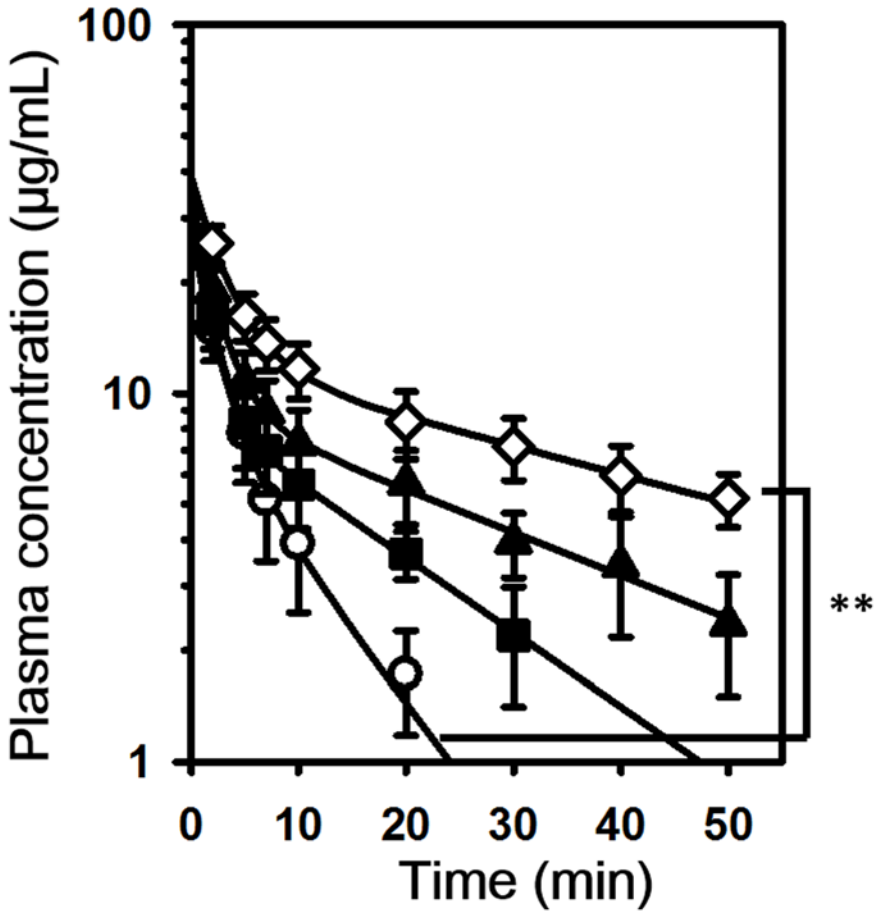
230

231 Fig.3 Plasma concentration profiles of 4NP at a dose of 2 mg after its i.v. administration

232 to rats at 37°C (○), 34°C (■), 32°C (▲), or 28°C (◇). Each point represents the mean ±

233 S.E. of at least five experiments.



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