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A Mouse Model for Evaluating the Induction of Liver Glucose-6-Phosphate Dehydrogenase by Halothane

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Abstract : The effect of halothane anesthesia in different doses and a period of two weeks on glucose-6-phosphate dehydrogenase (G6PDH) activity of mouse liver were investigated. The mean and SD of G6PDH for liver of normal mice were 0.036±0.017 U/mg protein and 1.756±0.814 U/g liver, respectively. Although there was histopathological damage in the liver, liver weights in treated mice increased significantly compared to control animals. Increasing the dosage of halothane inhalation induced liver G6PDH enzyme activity (U/g liver) by 84%, 42% and 4%, showing an inverse relation with the dose of halothane (0.25, 0.50, 1.00 cc twice daily for two weeks). Although the Michaelis constant (Km) of the partially purified enzyme for glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP) were similar, the utilization of analogs as 2-deoxyglucose-6-phosphate (2d-G6P), galactose-6-phosphate (Gal-6P) and nicotinamide adenine dinucleotide (NAD) were different. The induced enzyme was more heat stable than the control.

Key Words : Halothane, G6PDH, mouse liver, induction, kinetic properties

Halotan ile Oluşturulan Karaciğer Glukoz-6-Fosfat Dehidrogenaz İndüksiyonunun Değerlendirilmesinde Bir Fare Modeli

Özet : Bu çalışmada, iki haftalık zaman periyodunda uygulanan farklı dozlardaki halotan anestezisinin fare karaciğer glukoz-6-fosfat dehidrogenaz (G6PDH) aktivitesine etkisi araştırılmıştır. Normal fare karaciğerinde G6PDH aktivitesinin ortalaması (±SD) 0,036±0,017 Ü/mg protein ve 1,756±0,814 Ü/g karaciğer olarak bulunmuştur. Anestezi uygulanan farelerin karaciğerlerinde histopatolojik hasar oluştuğu ve karaciğer ağırlıklarında da anlamlı artış olduğu saptanmıştır. Artan dozlarda (2 haftada günde 2 kez 0,25, 0,50, 1,00 cc) halotan inhalasyonunun %84, %42 ve %4 (Ü/g karaciğer) oranlarında karaciğer G6PDH aktivitesini azaltması, halotan dozu ile enzim aktivitesi arasında ters ilişki olduğunu göstermektedir. Kontrol ve anestezi uygulanan gruplarda, kısmi saflaştırılmış enzimin glukoz-6-fosfat ve nikotinamid adenin dinükleotid fosfat michaelis sabiteleri (Km) benzemekte iken, 2-deoksiglukoz-6-fosfat, galaktoz-6-fosfat ve nikotinamid adenin dinükleotid gibi substrat analoglarının kullanımı yönünden farklılık gösterdiği saptanmıştır. İndüklenen enzimin ısı stabilitesinin kontrolden daha fazla olduğu saptanmıştır.

Anahtar Sözcükler : Halotan, G6PDH, fare karaciğeri, indüksiyon, kinetik özellikler

Introduction

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) is the first and key regulatory enzyme of the pentose phosphate pathway. The main physiological role of G6PDH is to provide reduced nicotinamide adenine dinucleotide phosphate (NADPH), a compound necessary for a number of biosynthetic reactions, including fatty acid synthesis, and the reducing equivalents maintain the redox state of the cell. It is a 'housekeeping' enzyme constitutively expressed in all cell types and in all organisms thus far analyzed. However, there are tissues such as liver, adipose, lung and proliferating cells where G6PDH activity responds to external stimuli such as hormones, growth factors, nutrients and oxidative stress, thus showing adaptive capacity (1-4).

Halothane has been widely used as an inhalational anesthetic since 1957 and is metabolized in the liver as a lipophilic xenobiotic to hepatotoxic intermediates by monooxygenases using cytochrome P4502E1 system (CYP2E1). The free radicals generated during the

metabolism of halothane are quenched by the antioxidant system present in the living cell as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH-R), glutathione peroxidase (GSH-P), reduced glutathione (GSH) and eventually the final electron acceptor NADPH generated by G6PDH (5-8).

Since human and mouse G6PDH enzyme show homology (9-11), a mouse model could be used for evaluating the effect of halothane anesthesia on the induction of G6PDH enzyme.

Materials and Methods

Chemicals: Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G6P), 2-deoxyglucose-6-phosphate (2d-G6P), galactose-6-phosphate (Gal-6P), nicotinamide adenin dinucleotide (NAD), deamino-nicotinamide dinucleotid phosphate (dNADP), bovine serum albumin (BSA), (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)) and phenazine methosulfate (PMS) were obtained from the Sigma Chemical Co. DE-52 was from Whatman (Maidstone, England). All the other chemicals were analytical grade products of Merck (Darmstadt, Germany).

Animals: Male, non-inbred *Mus musculus* albino mice, 6-10 weeks old, weighing 31±2 g were obtained from the Medical Sciences Experimental Research Center of the University of Çukurova. They were fed a standard laboratory diet and tap water. Illumination was 12 hour light/dark cycle and room temperature was 22-24°C. The control group consisted of fifteen apparently normal mice and fifteen were anesthetized with halothane. The anesthetized group was subdivided into three subgroups (a, b and c) which were given halothane 0.25 cc, 0.50 cc and 1.00 cc respectively through inhalation over 30 minutes twice daily at 8.00 AM and 4.00 PM for a period of two weeks. The mice were weighed before sacrifice. The livers were quickly removed, weighed and homogenized with three volumes of ice-cold 0.25 M sucrose. The activity of G6PDH enzyme was measured in 105,000 x g supernatant.

Enzyme Activity: Activity was determined at 37°C in the homogenate according to the procedure of Beutler (12). The protein concentrations were measured using BSA as the standard (13). The specific activity of the enzyme was calculated for homogenate as U/mg protein and U/g liver.

Biochemical Parameters: Kinetic studies followed the procedure of the WHO scientific group. G6PDH from mouse liver was partially purified by ion exchange with DE-52 as the ligand. The steady-state kinetic parameters were obtained spectrophotometrically using a Shimadzu U.V. 260 instrument. Michaelis constants (Km) for G6P and NADP were determined in partially purified enzyme at 37°C. G6P concentrations ranged from 10 to 1000 µM and NADP concentrations from 0.8 to 30 µM in the Km studies. The utilization rates of 2d-G6P, Gal-6P, dNADP, and NAD were measured. The heat stability of G6PDH enzyme was determined after 10 and 20 minutes incubation at 46°C (14). Polyacrylamide gel electrophoresis (PAGE) was performed using 0.08 M Tris-HCl buffer, pH 8.0, at room temperature. Before electrophoresis, samples were dialyzed 2 hours at +4°C against running buffer. The active band was stained by the staining solution containing 0.3 mM PMS, 2.8 mM MTT, 2mM NADP and 6 mM G6P in 10 ml. The gel was destained in 10% acetic acid at room temperature (15). The protein concentration of the liver was determined in a Shimadzu UV 120-02 spectrophotometer following the procedure of Lowry using BSA as a standard.

Histopathology: The liver tissues were fixed in 10% formaldehyde and processed routinely. They were embedded in paraffin. Five µm sections were obtained, stained with Harris hematoxylene-eosin and examined under a light microscope (16).

Statistics: The SPSSX program was used for the Wilcoxon-Mann-Whitney rank sum test (U test). Results were expressed as the means ± standard deviation (SD).

Results

Whereas anesthesia had no significant effect on the total body weight ($p>0.05$) of the mice, it did increase the weight of the livers as shown in Table 1, although the increase was barely significant ($p<0.05$). Table 2 shows the effects of anesthesia on G6PDH activity in the liver. Figure 1 shows the results of the thermal inactivation studies in the control group and in the anesthetized group. The thermal inactivation properties of the control group and the anesthetized group were also significantly different. The relative utilization of NAD, 2d-G6P and Gal-6P was significantly different as shown in Table 3. PAGE of both the control and the anesthetized group gave a single homogenous band and no differences in electrophoretic mobility were noted.

Table 1. Liver weights of control and anesthetized mice.

Groups	Liver weights (g)				
	n	X±SD	max	min	U-test
Control	15	1.37±0.01	1.75	1.02	
(a)	5	2.79±0.66	3.93	2.23	p<0.05
(b)	5	2.13±0.54	2.98	1.53	p<0.05
(c)	5	1.54±0.26	1.99	1.31	p>0.05

p<0.05 was considered significant

In groups a, b and c 0.25 cc, 0.50 cc and 1.00 cc of halothane, respectively, were given twice daily for two weeks.

Table 2. G6PDH activities of liver of control and anesthetized mice.

Groups	G6PDH activities				
	n	U/mg protein	U-test	U/g liver	U-test
Control	15	0.036±0.017		1.756±0.814	
(a)	5	0.052±0.037	p<0.05	3.239±0.756	p<0.05
(b)	5	0.039±0.011	p>0.05	2.498±1.193	p<0.05
(c)	5	0.028±0.008	p<0.05	1.840±0.832	p>0.05

p<0.05 was considered significant

In anesthetized groups a, b and c 0.25 cc, 0.50 cc and 1.00 cc halothane, respectively, were given twice daily for two weeks.

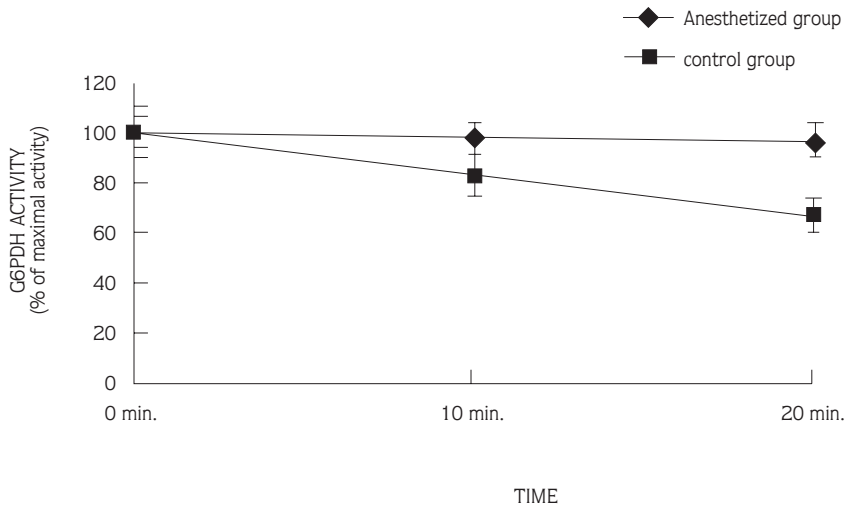

Figure 1. Thermal inactivation of mouse liver G6PDH enyzme of control and anesthetized mice.
The partially purified enzyme was incubated at 46°C. The G6PDH activity was measured at 0, 10, 20 min intervals.

Table 3. Biochemical properties of partially purified liver G6PDH of control and anesthetized (c) group.

Groups	Km		Utilization (%)				Heat stability (% activity remaining after 20 min. Incubation 46°C)
	G6P (μ M)	NADP (μ M)	2d-G6P	Gal-6P	dNADP	NAD	
Control	66.1±23.7	1.8± 0.2	4±8	0	48.0±9.8	57±34.7	69.0±10.1
Anesthetized	63.4±11.4	2.1± 0.9	*14±5.9	* 3.0±3.7	5.2± 3.3	*73 ±11.9	*98.7±6.3

* Significantly different (p<0.05) from the value obtained with control
In group c, 1.00 cc of halothane was given twice daily for two weeks.

Microscopic examinations of the liver tissues showed eosinophil leukocyte and mononuclear inflammatory cell infiltration on portal tracts. Cloudy degenerations, congestions, micro- and macrovesicular fatty degeneration, spotty necrosis and centrilobuler necrosis of the parenchymal cells were observed (Figure 2).

Discussion

Whereas the mechanism of halothane (2-bromo-2-chloro-1-1 trifluoroethane) anesthesia is still disputed, it is well established that the xenobiotic is metabolized in the liver through the P4502E1 system (CYP2E1) to

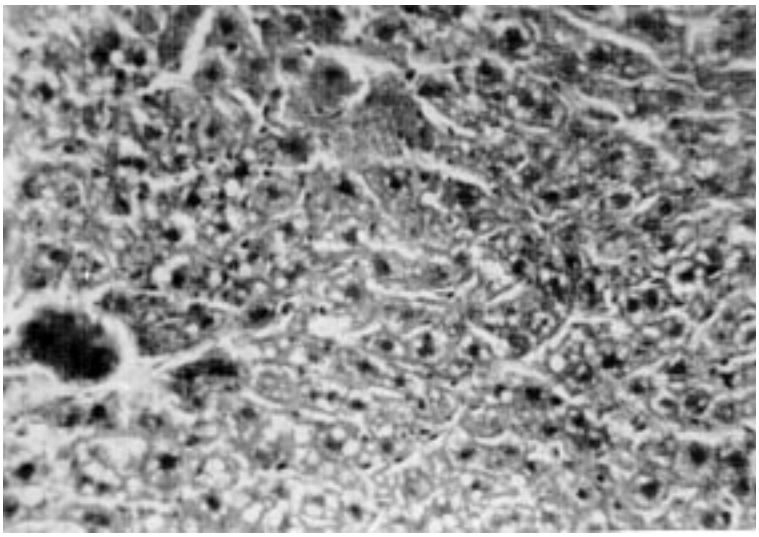

Figure 2. The tissue taken from the anesthetized group of *Mus musculus* albino mouse (Hematoxylene Eosin x375).
Liver tissue showing congestion, cloudy swelling, micro and macro vesicular fatty degeneration.

hepatoxic intermediates. The highly reactive free radicals generated during the course of the reaction are detoxified by GSH and/or by the final electron acceptor NADPH produced by the G6PDH enzyme (5-8, 17). In this study, also, we observed the toxic effects of long-term exposure to halothane anesthesia on the liver. As shown in Figure 2, the livers of mice suffered histopathological damage, changes in liver morphology were prominent and degeneration and necrosis of parenchymal cells were common. However, even after two weeks of anesthesia the livers increased in weight although no increase in body weight was observed. The increase in weight was significant and greatest in the low-dose anesthetic thus showing an inverse relation to the dosage of halothane. Even with the highest dose (1.00 cc twice daily for 2 weeks) the weight of the livers was greater compared to the control group. Similar observations were noted with the effect of ozone in the lung (18). Our most significant observation was the response of G6PDH enzyme to the xenobiotic halothane. The liver G6PDH enzyme also showed an inverse relation to the dose of halothane; the increase in enzyme activity was greatest with the low dose and decreased as the dose was increased. As with the liver weights, even with the highest exposure dose of halothane, the enzyme activity was higher than that of the control group. Similar inductions of mouse liver G6PDH were noted with exposure to phenylhydrazine and aflatoxin over a period of time (19,20). However, although the inductions of the enzyme were observed, no biochemical characterization of the enzymes was performed. In our experiments we partially purified and characterized the enzymes obtained from the livers of mice exposed to the highest halothane dose. The Michaelis constant (Km) of the substrate G6P and cofactor NADP were similar in the control and the anesthetized mouse liver. However, the utilizations of the analogs as 2d-G6P, Gal-6P and NAD were significantly different. Also, the heat stability of the induced enzyme was significantly changed, the latter being more stable. These data suggest that a variant of G6PDH enzyme is induced. Repression of G6PDH enzyme was first obtained by Pretsch et al. who first showed an X-linked deficient G6PDH enzyme in the offspring of mice by inbreeding normal mice with 1-ethyl-1-nitrosurea-treated male mice. The authors demonstrated that the repressed G6PDH enzyme was a variant of normal liver G6PDH by biochemical characterization. Similar variant results were obtained by Neifer et al., who characterized the variant obtained from 1-ethyl-1-nitrosourea-treated mice (21,22). However, no one has yet analyzed for the mutation in the gene. As is well known some variants have the same mutation although they differ in kinetic properties and vice versa (2,23,24). All these data support the model for the positive and negative effects (manipulations) suggested by Kletzien for the control of the G6PDH gene expression (4).

Conclusions

1. G6PDH enzyme was successfully used as a biomarker for the development of the hepatocellular defense against halothane.

2. G6PDH enzyme is induced in the liver although the livers suffered injury.

3. The induced enzyme had more heat stability and could use analogs as well.

4. Given the homology between human and mouse G6PDH, and since the mouse model is easy to manipulate, the G6PDH enzyme can successfully be used as a biomarker for the effect of different xenobiotics, drugs or carcinogens on the liver.

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